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PAPER

Severe tissue trauma triggers the autoimmune state systemic lupus erythematosus in the MRL/++ lupus-prone mouse

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Tissue damage associated with a severe injury can result in profound inflammatory responses that may trigger autoimmune development in lupus-prone individuals. In this study, we investigated the role of a large full-thickness cutaneous burn injury on the early onset of autoimmune disease in lupus-prone MRL/++ mice. MRL/++ mice (chronic model) exhibit autoimmune symptoms at >70 weeks of age, whereas MRL/-Fas^{lpr} mice (acute model) develop autoimmune disease in 17–22 weeks due to a lymphoproliferative mutation. Autoimmune disease developed in MRL/++ mice (4–15 weeks post injury) is manifested by skin lesions, vasculitis, epidermal ulcers, cellular infiltration, splenomegaly, lymphadenopathy, hypergammaglobulinemia, elevated autoantibodies and renal pathologies including proteinuria, glomerulonephritis and immune complex deposition; complications that contribute to reduced survival. Transcription studies of wound margin tissue show a correlation between the pathogenic effects of dysregulated IL-1β, IL-6, TNF-α and PGE_2 synthesis during early wound healing and early onset of autoimmune disease. Interestingly, MRL/++ mice with healed wounds (30–40 days post burn) strongly rejected skin isografts. Conversely, skin isografts transplanted onto naive age-matched MRL/++ littermates achieved long-term survival. Collectively, these findings suggest that traumatic injury exacerbates inflammatory skin disease and severe multi-organ pathogenesis in lupus-prone mice. *Lupus* (2009) **18,** 318–331.

Key words: autoimmunity; burns; lupus; SLE; trauma

Introduction

Systemic lupus erythematosus (SLE) is a chronic, complex autoimmune disease characterized by high levels of non-organ-specific, self-reactive antibody production directed against cellular, DNA, RNA and histone components leading to immune complex deposition.[1,2] The etiology of this inflammatory autoimmune disease remains elusive. The disease results in multiple health problems including increased infection, renal and skin disorders, neurological complications, osteoporosis, rheumatoid arthritis, osteoarthritis and fibromylagias.[3] A high morbidity and mortality rate is associated with SLE.[4]

Exposure to a number of environmental factors has been linked to the incidence of SLE. Moreover, not all individuals who carry disease-associated genes develop SLE; therefore, disease manifestation may be dependent on a complex array of environmental and genetic factors. Extreme physical and emotional stress, psychosocial and hormonal factors have been implicated as triggers for SLE.[2,5–7] Such factors have been linked to the manifestation of Gulf War Illness, a lupus-like condition.[8–13] Furthermore, exposure to chemicals, vaccines, medications, UV radiation and other ubiquitous environmental factors have been implicated in the induction of lupus-like disease in individuals with a genetic predisposition.[14–17] A number of studies suggest that the immune response to infectious agents and foreign antigens (bacterial, viral and allergen) play a key role in triggering activation of autoreactive T and B lymphocytes and inducing anti-DNA responses.[18–20]

Severe tissue trauma is a leading cause of disease experienced by military personnel and as accidents in civilian populations. The time course of wound healing depends on several factors including the type of wound, the extent of the tissue damage, inflammation, the presence of devitalized tissue and nonviable

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foreign tissue and infection. The immune system responds to a traumatic tissue injury by rapidly producing proinflammatory mediators, a response that is typically followed by a counteractive inflammatory response associated with profound and prolonged injury-induced immunosuppression. This counteractive response is thought to be protective in minimizing injury-induced inflammation while augmenting tissue repair. The wound healing response to a severe dermal injury is composed of multiple cellular and extracellular events.[21–24] Prolonged inflammation associated with severe tissue injury can result in additional tissue damage and profound immune dysfunction.[25,26]

MRL/++ mice have the same genetic background as MRL/-Fas^{lpr} mice but lack the lpr mutation and therefore develop renal disease at a later stage in their life (second year).[27–29] In this study, we show accelerated development of lupus-like autoimmune disease in young adult, wild type MRL/++ mice following severe tissue trauma (cutaneous burn wound). Burn-wounded MRL/++ mice develop early onset of severe SLE (10–15 weeks post injury), with characteristic skin lesions, cellular infiltration, hypergammaglobulinemia, anti-DNA autoantibodies, immune complex formation, glomerulonephritis and lymphadenopathy. Our results also show a correlation between the pathogenic effects of dysregulated cytokine production (IL-1β, IL-6, TNF-α, PGE2) and the early onset of SLE. We show that traumatic injury exacerbates inflammatory skin disease and the early onset of severe multiorgan SLE pathogenesis in lupus-prone mice.

Materials and methods

Animals

Five to six-week-old female MRL/++ mice and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) and housed in pathogen-free animal facilities at the Armed Forces Radiobiology Research Institute (AFRRI, Bethesda, MD USA) and the Walter Reed Army Institute of Research (WRAIR, Silver Spring, Maryland, USA), which are both accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. All procedures were conducted using facilities and protocols approved by the Animal Care and Use Committee of AFRRI (#2004-02-001) and WRAIR (protocol #K06-05). Mice were housed five animals per cage before surgery or any treatment and individually caged post-burn injury in standard micro-isolator polycarbonate caging. Mice were used for experimentation at 8 to 12 weeks of age. Animal rooms were maintained at 21° ± 2 °C with 50% ± 10% humidity on a 12-h light/dark cycle. Commercial rodent ration (Harlan Teklad Rodent Diet 8604) was available freely, as was acidified (pH = 2.5) water to control opportunistic infections.

Experimental design

At 12 weeks of age, MRL/++ mice received either a 15% full-thickness total body surface area (TBSA) burn or were sham-treated. Two sets of experiments were conducted. In the first set of experiments (n = 21 mice per burn-injured and sham-treated groups), we assessed the survival rate, urine proteinuria and the development of 'lupus-like' cutaneous lesion formations on the ears, neck and dorsum until the mice reached 9 months of age (6 months post injury). At days 1, 3 and 7 post injury, skin biopsies from another cohort of mice (n = 3 mice per group at each time point) were excised from the wound margin and screened using custom-made RT-PCR microarrays (Applied Biosystems Foster City, California, USA) containing oligo sequences for 184 inflammatory cytokine and wound repair gene transcripts. Mice that developed severe skin lesions and/or those with proteinuria levels of >500 dm/dL were euthanized by CO_2 inhalation followed by cervical dislocation. Immediately, post euthanasia, blood samples were collected by cardiac puncture for examination of serum IgG levels. Spleen and kidneys were removed to evaluate splenomegaly and immunopathology, respectively. Skin lesions and adjacent normal skin were excised, fixed with 10% formalin, embedded in paraffin and sectioned, 5 μm section per slide. The slides were deparaffinized and rehydrated and washed (3×) with phosphate-buffered saline solution (PBS) and stained with haematoxylin and eosin (H&E). In the second set of experiments (n = 5 mice per group), isogeneic skin graft experiments were conducted on mice 30–40 days either post-burn injury or sham-treatment. Skin graft survival was examined three times a week for 1 month. Photographs of skin lesions, skin grafts and histological sections were taken with a digital Fuji Finepix Camera or a Nikon DXM 1200 Digital Camera mounted on a Nikon Eclipse E800 microscope. Images were imported into Adobe Photoshop CS2 for reproduction.

Burn injury model

Mice were anaesthetized using either an intraperitoneal injection of ketamine (75 mg/kg), xylazine (15 mg/kg), acepromazine (2.5 mg/kg) or isoflurane

inhalation. After shaving the dorsum, the exposed skin was washed gently with room temperature sterile water and prepped with Betadine (a 10% povidone–iodine solution for skin disinfection). The Betadine solution from the prepped area was wiped off using three series of sponge gauzes containing 70% isopropyl alcohol. In a few selected studies, mice were further treated with a depilatory agent (Nair, Church and Dwight Co. Inc, Princeton, New Jersey, USA) to remove remaining hair stubble. Using a surgical skin marker, a 15-mm diameter circular area along the dorsal midline region was outlined. A full thickness burn (15% TBSA) was introduced with an electrocautery bovie (370–400 °C for 1.5 s: Bovie Aaron Medical, St. Petersburg, Florida, USA). This protocol causes a well-demarcated, full thickness injury in anaesthetized mice that is nonlethal with <0.5% mortality. Wounds became covered with eschar, and there was no macroscopic evidence of infection. Wounds were topically treated with triple antibiotic (Vetro-Biotic, Pharmaderm, Melville, New York, USA) immediately after burning and left uncovered without a dressing. Once mice recovered from anaesthesia, mice were housed in separate cages and maintained under standard conditions in the animal facility. With the exception of pain medication (Buprenorphine 0.1 mg/kg SC BID; Reckitt Benckiser Pharmaceuticals, Richmond, Virginia, USA) for the first two days post burn, no other treatment or topical wound care was administered. At various time points, post injury mice were euthanized by CO_2 inhalation followed by cervical dislocation.

Skin lesion, splenomegaly and lymphadenopathy assessments

Following either wounding or sham treatment, mice were observed weekly for skin lesions and protruding lymph nodes (cervical, brachial and inguinal). At the time of death or euthanasia, skin lesions were scored by gross pathology using the following scale: 0 = none, 1 = small and localized to one site (face or ears); 2 = moderate, more than one site involved, <2 cm (face, ears, dorsum) and 3 = severe, >2 cm (face, ears and dorsum). Spleens were weighed and enlarged lymph nodes scored on a scale of 0–3 (0 = none; 1 = small, at one site; 2 = moderate, more than one site and 3 = large, more than two sites).

Proteinuria

Urine was tested for proteinuria using commercially available kits (Multistix, Bayer, Elkhart, Indiana, USA). Proteinuria was scored as 0 (negative), <30 mg/dL (trace 0.5+), 30 mg/dL (1+), 100 mg/dL (2+) and >500 mg/dL (3+). Animals were considered to have proteinuria if they scored 2+ for two consecutive urine samples.

Serum Ig ELISA

Total serum IgG, IgG1, IgG2a, IgG2b and IgG3 isotype concentrations were determined by ELISA. Polystyrene plates precoated with goat anti-mouse Fc specific IgG capture antibody and blocked were commercially purchased (R&D Systems, Minneapolis, Minnesota, USA). One hundred microliters of Ig standards (Southern Biotechnology Associates, Birmingham, AL) was added per well in a series of two-fold dilutions (125 ng/mL–3.9 ng/mL), and serum Ig concentrations were assessed at a 1:200,000 dilution (100 µL per well). After 2 h of incubation at room temperature, the plates were washed three times with PBS containing 0.05% Tween-20 (wash buffer). Bound Ig was detected with 100 µL per well of appropriately diluted horseradish peroxidase conjugated anti-IgG (Chemicon, Temecula, California, USA), IgG1, IgG2a, IgG2b and IgG3 antibodies (Southern Biotechnology Associates, Birmingham, AL). Secondary antibodies were added to the plates and kept for 1 h at room temperature, followed by three washes with wash buffer. Then, 100 µL per well of freshly prepared substrate solution containing equal volumes of 0.4 g/L 3,3′,5,5′ tetramethylbenzidine and 0.02% hydrogen peroxide was used to develop the assay (Pierce, Rockford, Illinois, USA). Reaction was stopped with 100 µL per well of 2 N sulphuric acid (Sigma, St Louis, Missouri, USA), and the absorbance was measured at 450 nm using a 680 Microplate reader (BioRad, Hercules, California, USA). Results are denoted as the Ig concentration (mg/mL) at various time points.

Anti-dsDNA Ab ELISA

Ig class-specific anti-DNA antibodies were measured by ELISA. Polystyrene covalink 96-well microtitre plates (Fisher, Pittsburgh, Pennsylvania, USA) were coated with 50 µL per well of 10 µg/mL Calf Thymus DNA (Sigma, St Louis, Missouri, USA) and allowed to incubate overnight at 4 °C. After washing three times with wash buffer, 300 µL of blocking solution (3% bovine serum albumin, BSA, in PBS) was added per well and incubated for 2 h at room temperature. The plates were washed three times with wash buffer, and 100 µL of diluted sera was added to each well, (dilutions ranged from 1:50 to 1:100,000). After 2 h of incubation at room temperature, plates were washed three times with wash buffer. Then, 100 µL

of appropriately diluted horseradish peroxidase conjugated anti-IgG (Chemicon, Temecula, California, USA), IgG1, IgG2a, IgG2b, and IgG3 antibodies (Southern Biotechnology Associates, Birmingham, AL) was added per well to the plates for 1 h and followed by three washes. One hundred microliters per well of freshly prepared substrate solution containing equal volumes of 0.4 g/L 3,3′,5,5′ tetramethylbenzidine and 0.02% hydrogen peroxide were used to develop the assay (Pierce, Rockford, Illinois, USA). Reaction was stopped with 2 N sulphuric acid (Sigma, St Louis, Missouri, USA), and the absorbance was measured at 450 nm using a 680 Microplate reader (BioRad, Hercules, California, USA). Results are denoted as the OD_{450} at various dilutions.

Renal histopathology

Mice were euthanized by CO_2 inhalation followed by cervical dislocation, and the kidneys were removed. One kidney was fixed with buffered formalin for >48 h, embedded in paraffin blocks, sectioned and stained with haematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) by standard methods. Glomerular pathologies were evaluated morphometrically by light microscopy. The glomerular lesion (mesangial hypercellularity, increase in mesangial matrix, crescent formation and necrosis) was graded on a semiquantitative scale from 0 to 3 (0 = normal, 1 = mild, 2 = moderate, 3 = severe) for more than 20 glomeruli per mouse. Scores assigned to each of these elements were added together to yield a mean renal score. Values were reported as the mean ± standard deviation (SD) of seven specimens. For immunofluorescence studies of deposition of Ig's, the second kidney was embedded in optimal cutting temperature (OCT) compound (Miles Inc, Elkhart, Indiana, USA) and snap-frozen in a solution of 2-methylbutane and dry ice. Tissue samples were stored at −80 °C until further analysis.

Immunofluorescence and immunohistochemistry

Snap frozen kidneys were cut into 3-μm thick cryosections mounted on glass slides. A DAKO Autostainer Plus Universal Staining System (DAKO, Carpenteria, California, USA) was used for the immunofluorescent and immunohistochemical staining. Immunofluorescent detection of IgG was performed on sections of frozen blocks of mouse kidney using a FITC-conjugated goat-antimouse Ig antibody (Jackson Immunoresearch Laboratories Inc., West Grove, Pennsylvania, USA), incubated for 30 min at room temperature using a 1:250 dilution prepared with background reducing antibody diluent (DAKO) and visualized by dark field microscopy. Immunohistochemical detection of C3 was performed on sections of frozen blocks of mouse kidney using a labelled polymer (EnVision plus rabbit, DAKO, Carpenteria, California, USA) for visualization by light field microscopy. Rabbit polyclonal antibody for C3 (Abcam, Cambridge, Massachusetts, USA) was used at a dilution of 1:10 with background reducing antibody diluent (DAKO) and incubated for 30 min at room temperature. The chromogen 3,3′ diaminobenzidine (DAKO) was used. Sections were counterstained with haematoxylin (DAKO) and then cover-slipped. Negative tissue controls included normal mouse kidney. Negative reagent controls consisted of a serial section (the second unstained frozen slide), processed identical to the first unstained frozen slide, but normal rabbit serum was substituted for the primary antibody in every assay.

RNA extraction

Mice were euthanized by CO_2 inhalation followed by cervical dislocation on days 1, 3 and 7 post-burn injury. Total RNA was extracted from skin excised from the wound margin and stored in RNAlater (Ambion, Austin, Texas, USA). Briefly, skin tissue was homogenized in Trizol reagent (Invitrogen, Carlsbad, California, USA), and total RNA was isolated using Qiagen RNeasy Lipid Tissue Mini Kit (QIAGEN Inc., Valencia, California, USA) according to manufacturer's instructions. RNA was resuspended in 30 μL of 10 mM Tris buffer, pH 7.5. Sample purity, quantity and quality were assessed by determining the $A_{260/280}$, $A_{260/230}$ ratio on a Nanodrop-100 Spectrophotometer (NanoDrop Technologies Inc. Wilmington, Delaware, USA) and by measuring 28S/18S ribosomal RNA ratio and RNA Integrity Number (RIN) using an Agilent 2100 BioAnalyzer (Agilent Technologies Inc. Santa Clara, California, USA). All Agilent RNA integrity values were ≥8.5. Reverse transcription was performed with Roche 1st Strand Synthesis kit (Roche Diagnostics Corporation, Indianapolis, Indiana). Briefly, 2.5 μg of RNA sample was added to a master mix containing 1X reaction buffer, 5 mM $MgCl_2$, 1 mM deoxynucleotide mix, 6.4 μg random primers, 100 units RNase inhibitor and 40 units Avian myeloblastosis virus transcriptase. Ten millimolar Tris buffer, pH 7.5, was used to reach 40 μL final reaction volume. Then, final reaction mixture was subjected to a single reverse transcription cycle of 25 °C for 10 min, 42 °C for 60 min, 99 °C for 5 min and 4 °C for at least 10 min.

Real-time quantitative PCR (RT-PCR) gene profiling for proinflammatory transcripts

Quantitative real-time polymerase chain reaction (RT-PCR) was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, California, USA). Custom-designed 'Wound Repair' TaqMan® Low Density Array (TLDA) cards (Applied Biosystems, Foster City, California, USA) were used to assess gene expression. The set of TLDA cards were composed of 184 individual target genes [including respective forward and reverse primers and a dual labeled probe (5'-6-FAM; 3'-MGB)] in quadruplicate on a 384-well card (96 genes per card). Amplification parameters were as follows: one cycle of 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. Two samples were processed on each card.

RT-PCR data analysis

RT-PCR data were analyzed using the Sequence Detection System version 2.1 included with the ABI Prism 7900HT SDS and using Microsoft Excel. The threshold cycle (C_t) for each sample was manually set to 0.2 and the baseline was set between 3 and 15 cycles. 18S ribosomal RNA was used as an endogenous housekeeping control gene for normalization, and the comparative C_t method was used to calculate the relative fold expression by $2^{-\Delta\Delta C_T}$.[30,31] Assays with C_t values greater than 35 cycles were excluded from analysis.

Skin isograft transplantation

Mice were transplanted with skin isografts, as described elsewhere.[32] Briefly, full-thickness skin grafts (3×3 cm^2) were obtained from the flanks of naive donor MRL/++ mice and transplanted onto the dorsal flanks of syngeneic naive (uninjured) and experimental recipient female MRL/++ mice which had fully recovered from a previous 15% full-thickness TSBA burn injury (15–17 weeks of age). Grafts, 3 cm^2 in area, were fitted to the prepared bed without suturing and then covered with an adhesive plastic bandage. After 7 days, the adhesive bandage was removed. Graft survival was then followed by daily visual inspection. Rejection was defined as complete necrosis and loss of viable skin tissue.

Statistical analyses

Mann-Whitney's *U*-test was used to determine the statistical significance of differences between groups. Survival, incidence of proteinuria and skin graft rejection–survival were analyzed by the Kaplan–Meier method, and the Log–rank test was used to determine the statistical significances. *P* values less than 0.05 were considered significant.

Results

Severely injured MRL/++ mice develop a 'lupus-like' syndrome

Within 1–2 months after burn injury (4–5 months of age), 57% of the MRL/++ mice with healed wounds began to exhibit a lupus-like phenotype characterized by severe, excoriating dermatitis–vasculitis in the dorsum and scapular regions ± ear necrosis (Figure 1A–C). Histological sections of MRL/++ skin lesions show mixed acute and chronic inflammatory cell infiltrates extending from the epidermis to the subcutis with abnormal hair follicle proliferation (data not shown). On the contrary, no such lesions were observed in sham-treated MRL/++ mice, sham-treated BALB/c mice and burn injured BALB/c mice.

The development of urine protenuria is a key factor in the progression of renal disease in lupus-prone mice. Following sham-treatment and burn injury, we monitored urine protein levels on a weekly basis as an index of proteinuria. Mice were considered to have proteinuria if they scored >100 mg/dL (>2+) for two consecutive urine samples within a 2 week timeframe. The cumulative incidence of proteinuria (>100 mg/dL) for each group of mice is shown in Figure 2A. The incidence and severity of urinary protein scores (Figure 2B) increased in injured MRL/++ mice over time compared with sham-treated MRL/++ mice, wherein only 1 mouse developed severe proteinuria at day 135 post-treatment. On the contrary, minimal protein levels were detected in the urine collected from burn-injured or sham-treated BALB/c mice throughout the study interval (data not shown).

Over the course of the study, the percentage of burn-injured MRL/++ mice that developed significant skin lesions and proteinuria increased over time (Figure 1C). The difference in survival was even more striking. MRL/++ mice presenting with SLE lupus-like syndrome died significantly earlier, with a median survival rate of 103 days, when compared with the 100% survival of sham-treated BALB/c mice during the 6-month evaluation time period. Six months after the severe burn injury (8 months of age), only 2 of 21 of the original burned MRL/++ mice were alive with no gross macroscopic evidence of cutaneous autoimmune disease. As depicted in Figures 2C, a biphasic survival response ensued with a cohort of

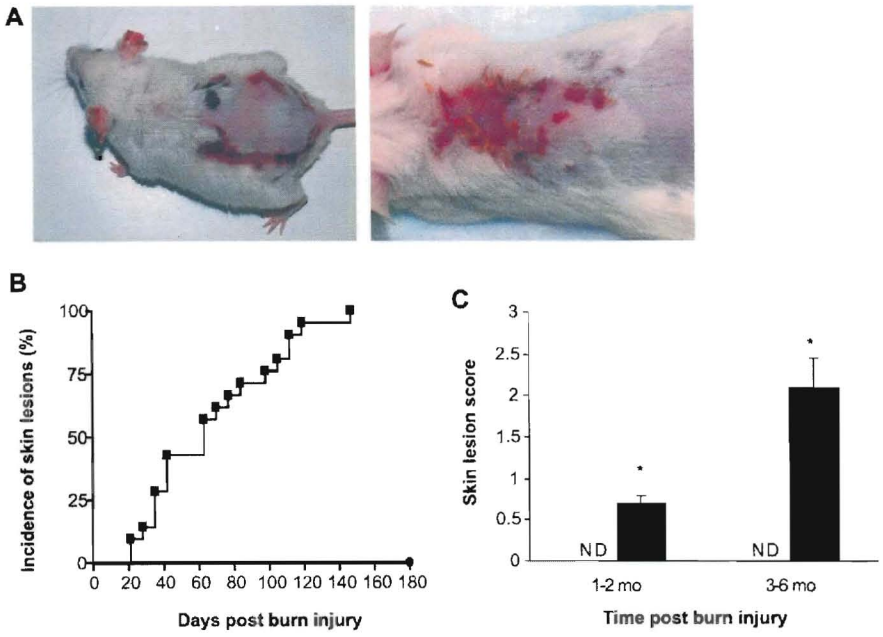

Figure 1 Burn trauma augments SLE development in lupus-prone MRL/++ mice. (A) Photographs of typical skin and ear lesions in MRL/++ mice exhibiting lupus-like symptoms at 2–6 months post-burn injury. On the contrary, no lesions were observed in burned BALB/c mice or age-matched, sham-treated MRL/++ mice. (B) Cumulative incidence burn-injured MRL/++ mice [■] and sham-treated MRL/++ mice [•] exhibiting skin lesions. Results presented as a Kaplan–Meier plot ($n = 21$ mice per group, $P < 0.05$). (C) Mean skin lesion score (*see Material and Methods*) of burn-injured mice [■] and sham-treated mice [□]. ND = not detectable. $^*P < 0.05$, burn-injured compared with sham-treated mice.

MRL/++ mice that displayed autoimmunity within 1–2 months post-burn injury and a separate cohort of MRL/++ mice that developed cutaneous lupus-like lesions 3–6 months post-burn injury. For this reason, data from these two groupings were pooled and evaluated separately. In sharp contrast, 19 of 21 sham-treated age-matched control MRL/++ mice survived to greater than 36 weeks of age and showed no incidence of cutaneous disease and minimal proteinuria during the same observation period. One sham-treated MRL/++ mouse spontaneously died at 3 months of age and another at 5 months of age with cause of death unknown. Sham-treated (21 of 21) and burn-injured BALB/c mice (21 of 21) appeared healthy throughout the study period, showing no signs of proteinuria or premature death and thus were not evaluated rigorously. Notably, at the time of euthanasia, the comparison of spleen weights between wounded MRL/++ mice at 4–6 months post injury and sham-treated mice at 6 months post injury showed a mild splenomegaly (~1.7-fold increase) in mice exhibiting lupus-like disease (393 ± 90 mg, $n = 10$ versus 231 ± 60 mg, $n = 13$, $P < 0.05$, Figure 3A). Similar increases were noted in the size of some of the cervical, brachial and inguinal lymph nodes. Approximately, 30% of the mice with skin lesions had enlarged lymph nodes at 4–6 months post-burn injury, whereas sham-treated MRL/++ mice did not exhibit visible signs of enlarged lymph nodes (Figure 3B). In comparison, no significant differences in spleen weight and lymph nodes size between sham-treated and wounded BALB/c mice were observed (data not shown).

Serum hypergammaglobulinemia and anti-DNA antibodies

Escalating hypergammaglobulinemia and elevated levels of serum autoantibodies, such as anti-dsDNA antibody, play a major role in the pathogenesis of autoimmune SLE-like disease in MRL/++ lupus-prone mice. To determine whether burn injury affected serum Ig concentrations in MRL/++ mice, we measured total serum IgG1, IgG2a, IgG2b and IgG3 antibody levels by ELISA at 0–2, 4–8, and 12–24 weeks post-burn injury and in sham-treated mice at 24 weeks (end of study). As shown in Table 1, burn injury in MRL/++ mice induced a significant elevation (up to threefold increase) of serum IgG1, IgG2a, IgG2b and IgG3 isotypes in comparison to Ig levels in

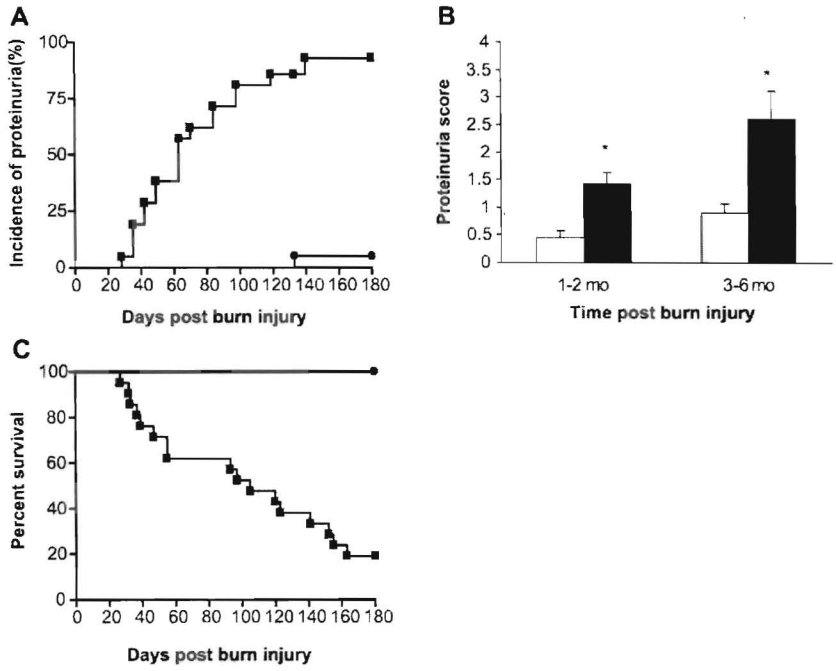

Figure 2 Wounded lupus-prone MLR/++ mice develop proteinuria and have marked decrease survival in comparison either to age-matched sham-treated MRL/++ mice or control BALB/c mice (data not shown). (A) Cumulative incidence of proteinuria (>100 mg/dL) (B) Mean proteinuria score (*see Material and Methods*) of burn-injured mice [■] and sham-treated mice [□]. (C) Percent survival rate of burn-injured MRL/++ mice (■) and sham-treated MRL/++ mice [●]. $*P < 0.05$, burn-injured MRL/++ mice compared with sham-treated MRL/++ mice.

the serum of sham-treated MRL/++ mice after 24 weeks of time. Interestingly, serum levels of circulating anti-double-stranded DNA antibodies (IgG2a, IgG2b and IgG3 isotypes) were significantly increased in burned injured MRL/++ mice at 12–24 weeks post wounding (Figure 4). In particular, the ratio of the anti-dsDNA IgG2a to anti-dsDNA IgG1, a parameter of Th1/Th2 balance, was significantly increased in wounded MRL/++ mice at 12–24 weeks post injury. Furthermore, the increased production of IgG3 is of particular importance as it has been considered a 'nephritogenic' Ig.[33] Notably, the frequency of IgG2a, IgG2b and IgG3 anti-dsDNA antibodies was significantly lower in sham-treated MRL/++ mice. As expected, no significant differences in serum IgG isotypes and IgG-specific anti-DNA antibodies were

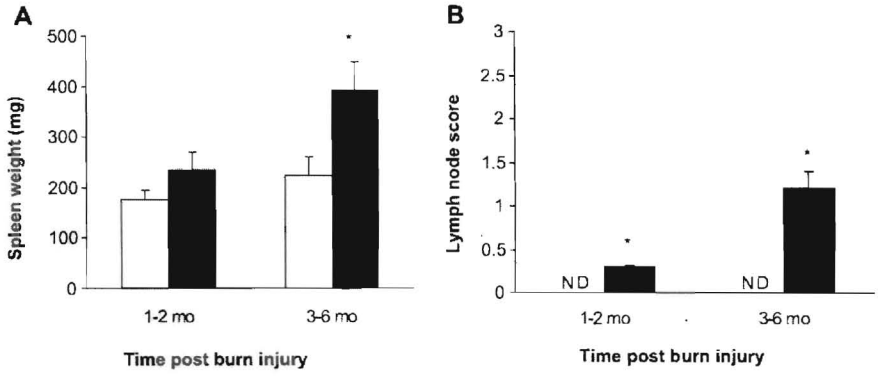

Figure 3 Spleen weights (A) and lymph node scores (B) in burn-injured mice [■] and sham-treated mice [□] MRL/++ lupus prone mice. ND = not detectable. $*P < 0.05$, burn-injured MRL/++ mice compared with sham-treated MRL/++ mice.

Table 1 Serum IgG subclasses in sham-treated controls and burned MRL/++ mice at the time of euthanasia[a]

	Sham-treated	*Burned*
IgG1		
0–2 weeks	ND	0.43 ± 0.259
4–8 weeks	ND	5.52 ± 0.77*
12–24 weeks	1.46 ± 0.18*	4.41 ± 1.88*
IgG2a		
0–2 weeks	ND	0.14 ± 0.13
4–8 weeks	ND	1.59 ± 0.34*
12–24 weeks	0.64 ± 0.29*	2.16 ± 0.51*
IgG2b		
0–2 weeks	ND	<0.014
4–8 weeks	ND	0.17 ± 0.6*
12–24 weeks	0.09 ± 0.03	0.27 ± 0.08*
IgG3		
0–2 weeks	ND	<0.01
4–8 weeks	ND	0.17 ± 0.06*
12–24 weeks	0.01 ± 0.01	0.27 ± 0.07*

Abbreviation: ND = not detectable.
[a]Sera IgG isotypes were measured by ELISA at 1:200,000 dilution. Results are expressed as the Ig concentration in mg/mL ± SEM.
*$P < 0.05$ versus 0–2 week post-burn measurements.

detected in either burn or sham-treated BALB/C mice at the end of the study period (data not shown). Collectively these findings indicate wound trauma promotes production of anti-dsDNA autoantibodies in lupus-prone mice.

Burn injury increases kidney Ig and C3 deposition

Glomerulonephritis is a well-defined and characterized pathological feature of murine SLE. To evaluate the effects of burn injury on renal pathology, kidney sections obtained at the time of necropsy were examined by standard histopathological and immunohistochemical techniques for evidence of glomerular inflammation and immune complex deposition. The photomicrographs in Figure 5A are representative glomeruli from a wounded MRL/++ mouse exhibiting lupus-like syndrome 90 days post injury and glomeruli from an age-matched sham-treated control MRL/++ mouse. PAS stained glomeruli from mice presenting with lupus-like syndrome typically showed a marked increase in glomerular cellularity with histopathological evidence of diffuse proliferative glomerulonephritis, segmented glomeruli, proliferative changes in mesangial and endothelial cells of the glomeruli, increase in mesangial matrix, capillary basement membrane thickening, mononuclear cell infiltrates in interstitium and often the presence of intratubular proteinaceous casts. All these findings are indicative of glomerular dysfunction. Kidneys from age-matched, sham-injured MRL/++ mice showed glomeruli with normal cellularity, mesangium and glomerular basement membranes.

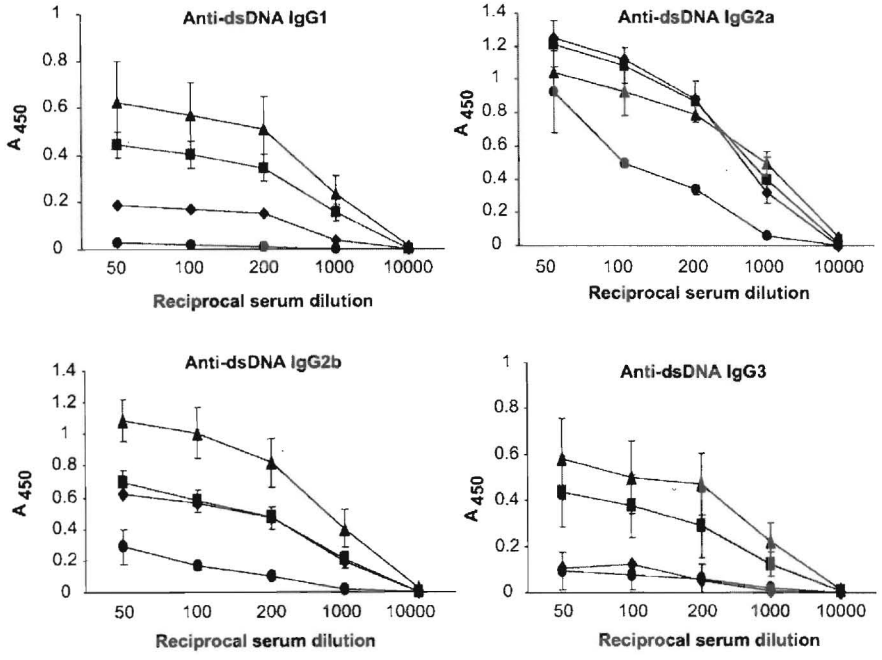

Figure 4 Serum anti-dsDNA antibody titre levels of different IgG subclasses between burn-injured MRL/++ mice (● 1–2 weeks post burn; ■ 4–8 weeks post burn; ▲ 12–24 weeks post burn) and sham-treated MRL/++ mice (♦ 24 weeks). Reactivity of diluted serum with calf thymus DNA was determined by ELISA. Values are the mean ± SD absorbance values at 450 nm (4–8 serum samples per time point).

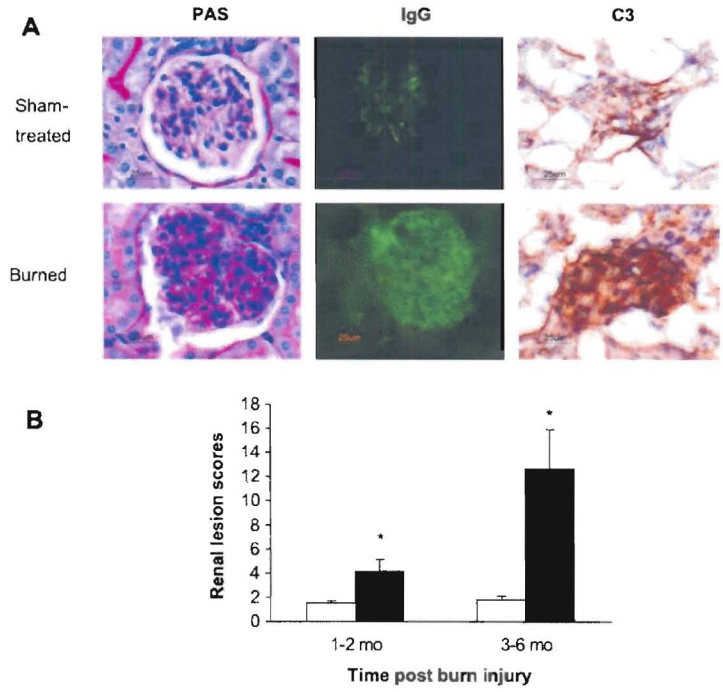

Figure 5 Accelerated glomerulonephritis and immune complex deposition in lupus-prone MRL/++ mice following burn-injury trauma. (A) At the time of death or euthanasia, the kidneys were removed and then sectioned before staining with PAS, FITC-conjugated anti-mouse IgG, or anti-mouse C3. Representative photomicrographs of glomeruli from burn-injured and sham-treated MRL/++ mice are shown (400× magnification; scale bars = 25 μm). In burn-injured mice, immunofluorescence for IgG was diffusely, globally and strongly presented as intraglomerular deposits in the mesangium and capillary wall. C3 staining was presented multifocally or globally as intraglomerular deposits in the cytoplasm of the mesangium and capillary wall. C3 deposits resulted in a thick appearance of the glomerular capillary loops. These IgG and C3 deposits resulted in a thick appearance of glomerular capillary loops. The level of Ig and C3 immunostaining was strong in comparison to age-matched, sham-treated mice. (B) Kidney sections were graded for glomerular inflammation, cellular infiltration, proliferation, crescent formation and necrosis. Scores from 0 to 3+ were assigned to each of these elements and then added together to yield a mean renal score (n = 5 mice; 10–15 glomeruli per kidney section were counted, 2–3 sections per mouse). *$P < 0.05$, burn-injured MRL/++ mice compared with sham-treated MRL/++ mice.

Collectively, the average renal lesion score in mice exhibiting lupus-like syndrome was significantly greater than that of uninjured age-matched control MRL/++ mice (Figure 5B). Glomeruli from sham-treated or burn-injured BALB/c mice showed no evidence of glomerular disease. Consistent with these observations, we detected intense glomerular deposition of total IgG in the peripheral capillary loops of the glomeruli from wounded MRL/++ mice by immunofluorescence staining. Similarly, immunostaining against C3 showed comparable immune complex deposition. Such deposits were found mainly within the affected glomeruli. In sharp contrast, immunofluorescence and immunostaining analysis of sham-treated MRL/++ kidneys showed minimal Ig and C3 deposition. Collectively, these findings suggest that wound trauma accelerates the onset of glomerulonephritis in lupus-prone mice.

Aberrant production of cytokine and PGE_2 mRNA transcripts at the wound margin post-burn injury in lupus-prone MRL/++ mice

Abnormalties in cytokine production have been shown to contribute to the development of autoimmune disease in lupus-prone mice. To determine whether accelerated lupus onset in burn-injured MRL/++ mice is related to aberrant expression of mediators that play a role in the early inflammatory response, we measured the transcript levels of 184 genes (cytokines, chemokines, growth factors, wound repair response mediators) using custom-made taqman cDNA arrays. It is interesting that transcripts levels for IL-1β, TNF-α and PGE_2 were generally higher earlier in the wound healing process in MRL/++ wound margin tissue (Figure 6) when compared with the expression levels of these mediators in the

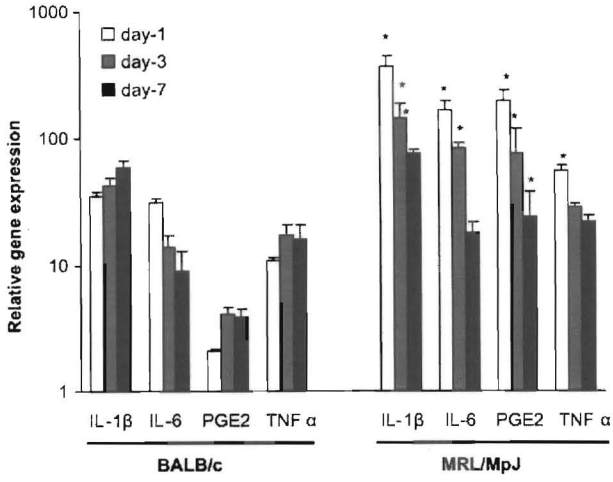

Figure 6 Quantitative analysis of IL-1β, IL-6, TNF-α and PGE-2 transcripts in MRL/++ and BALB/c wound margin skin tissue at days 1, 3 and 7 days post-burn injury. The results represent the mean ± SD ($n = 6$) relative gene expression level of transcripts in comparison to those levels present in naive skin. $^*P < 0.05$, burn-injured MRL/++ mice compared with burn injured BALB/c mice.

wound margins of burned BALB/c mice which did not develop cutaneous and renal pathologies. There was no significant difference in the expression of both Th1 and Th2 cytokines and other inflammatory gene transcripts between MRL/++ and BALB/c mice (data not shown).

Skin isograft rejection

To clearly determine that lupus-prone MRL/++ mice develop both humoral and cell-mediated arms of adaptive autoimmunity (loss of tolerance to self antigens) following wound healing, we evaluated whether these mice could mount a rejection response to a transplanted 'self' skin (isograft). We transplanted syngeneic naive skin onto the dorsum of MRL/++ mice 30–40 days post-burn injury. Graft survival was determined and compared with that of non-injured, age-matched control MRL/++ mice. As shown in Figure 7, MRL/++ mice that were previously subjected to severe wound trauma promptly rejected the naive MRL/++ syngeneic skin, with a mean survival time of 8 days ($n = 5$). Histological analysis of the isografts showed heavy lymphocytic infiltration and extensive tissue damage (data not shown). On the contrary, skin graft sites ($n = 5$) on sham-treated MRL/++ mice were uniformly healed by 2-weeks post transplantation. The graft integrity remained intact throughout the 30-day observation period without any gross visible evidence of rejection. Microscopic evaluations of these grafts failed to identify any inflammatory lesions and showed normal epidermis and dermis architecture (data not shown).

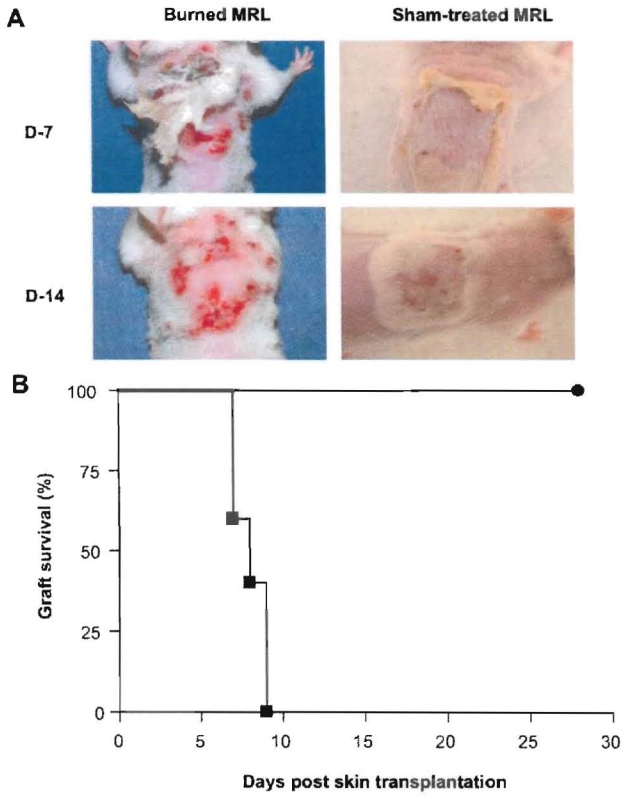

Figure 7 Burn injury in lupus-prone MRL/++ mice results in the loss of tolerance to self. Severely injured MRL/++ mice reject skin isografts from naïve MRL/++ donors whereas skin grafts were uniformly healed within 2-weeks and accepted for more than 30 days after transplantation in sham treated age-matched control MRL/++ recipient mice ($n = 5$). (A) Photographs of skin isografts at day 7 and 14 post transplantation. (B) Graft survival was determined and presented as a Kaplan-Meier plot ($n = 5$ per group, $P < 0.05$). Burn injured MRL/++ mice (■) and sham-treated MRL/++ mice [●].

Discussion

Our findings show that severe trauma can contribute to autoimmune disease progression in MRL/++ lupus-prone mice. We show that a severe burn injury accelerates the development of severe skin lesions, vasculitis, lymphoadenopathy, hypergammaglobulinemia, circulating autoantibodies and renal disease pathology (including proteinuria, IgG and C3 deposits and glomerural basement thickening) compared with age-matched, sham-injured MRL/++ mice. The differences in the rate of disease progression and

survival were even more striking. The early and pronounced increase in serum autoantibodies is most likely a key factor contributing to accelerated disease occurrence and correlated with disease severity. The early development of both humoral and cell-mediated arms of adaptive autoimmunity (loss of tolerance to self antigens) during and immediately following the wound healing responses was demonstrated as a result of rapid rejection of transplanted 'self' skin isografts before any evidence of phenotypic disease. These data suggest that severe trauma can be added to the list of triggering events that promote the manifestation of SLE autoimmune disease in lupus-prone MRL/++ mice.

SLE is a complicated inflammatory process characterized by the interactions of components of both adaptive and innate immunity.[34] Severe injury has been shown to lead to pronounced defects in immune function, including increased proinflammatory cytokine production, decreased antigen recognition, increased Th2 cytokine production and altered antibody production.[25,35–41] A complex interplay of multiple inflammatory mediators, including leukocytes, cytokines, chemokines, adhesion molecules, complement, as well as antibodies is thought to play a major role in the progression of autoimmune SLE disease.[39,42–46] Despite strain differences in expression, we found that mRNA transcripts for IL-1β, IL-6, TNFα and PGE_2 transcripts in comparison to IL-1α, IL-2, IL-4, IL-5, IL-10 and TGF-β transcripts were consistently more pronounced earlier in MRL/++ wound margin tissue in comparison to BALB/c wound margin tissue. These potent factors mediate the systemic effects of inflammation after a severe burn injury and are produced primarily by infiltrating activated macrophages in response to infectious or immune signals and have direct stimulatory effects on T and B lymphocytes, natural killer cells (NK), dendritic cells (DC) and myeloid cells by enhancing their proliferation, activation and survival.[47–51] During severe traumatic injuries, like many autoimmune diseases, the production of these cytokines is dysregulated and contributes to macrophage hyperstimulation,[49] wherein macrophages become globally inhibitory and induce elevated production of IL-10, which enhances Th2 responses.[48–50,52] These activated innate immune cells play a major role in SLE autoimmune diseases, as antigen-presenting cells and primary effector cells that cause tissue damage and loss of kidney function.[34] Interestingly, Voronov, *et al.*,[53] and Liang, *et al.*,[54] reported that IL-1β-deficient mice and anti-IL-6 antibody-treated mice are resistant to SLE induction, respectively. Our findings are consistent with studies suggesting potent local and systemic roles for proinflammatory mediators (IL-1β, IL-6, TNFα) in promoting the differentiation of Th2 autoimmune responses in both SLE patients and lupus-prone mice.[44–46,55–62]

Autoimmunity coincides with the loss of tolerance to the self[3,19]; it is thought of as a persistent failure of an integrated fabric of components rather than the adverse consequence of a 'specific forbidden clone'.[63,64] In comparison to uninjured MRL/++ mice, where skin acceptance and healing in the gross appeared to be complete at 14 days with a full pelt of hair by day 21, syngeneic skin grafts in previously wounded MRL/++ mice were uniformly rejected in 7–10 days. It is unclear whether the antigen-driven isograft rejection response is T-cell or B-cell (humoral) mediated or both, although we detected high serum levels of autoimmune antibodies. Elevated production of Th2-dependent Ig autoantibody subclasses in the serum of wounded MRL mice strongly suggests a skewing of the Th1/Th2 balance toward a Th2 response. MRL/++-wounded mice had significantly elevated serum levels of anti-dsDNA antibodies of the IgG1, IgG2b and IgG3 isotypes, isotype switching which is known to be dependent on Th2 cytokines.[59,60] Our results are consistent with the model that glomerulonephritis in autoimmune kidney disease is predominantly dependent upon IgG2b and IgG3 Th2-dependent nephritogenic autoantibodies deposition.[65,66]

Although not an aim of our current study, sufficient preceding work reports that the clinical course of SLE is frequently associated with an acquired hypercoagulation state, involving elevated and persistent serological levels of antiphospholipid (aPL) antibodies.[67,68] Such events can lead to arterial, venous and microcirculatory thrombotic complications resulting in accelerated disease manifestation and life-threatening thrombotic events.[69] The reported early occurrence of aPL antibodies before SLE diagnosis in patients with no evidence of underlying disease[70] suggests that a primary antiphospholipid syndrome (PAPS) may be an important predictor of SLE development[67,68]; wherein prothrombin and β_2-glycoprotein (β_2-GPI) represent the major target antigens for lupus anticoagulant and anti-cardiolipin aPL antibodies, respectively.[67,68] Moreover, the numerous cutaneous pathologies evident in SLE patients are indicative signs of thrombotic events that can occur before, simultaneously and after the onset of life-threatening thrombotic events.[68,70] In this regard, there has been much focus in assessing inflammatory and noninflammatory events that affect vessel wall endothelial cell activation/damage and increased platelet aggregation, which may con-

tribute to vasculitis and vasculopathy processes in arterial and venous vessels.[71,72] Although beyond the scope of the current study, it would be important and of clinical relevance to show whether wound trauma in lupus-prone mice also promotes a thrombophilic state. Follow-on investigations to determine the profile and developmental kinetics of known thrombophilic factors including lupus anticoagulant, anticardiolipin and anti- β_2-GPI will help to define the interrelations between severe tissue injury, exacerbated inflammatory reactions, serological abnormalities and SLE development pathogenesis.

The environmental signals–mediators that trigger the early onset and development of autoimmune disease following severe traumatic injuries remain to be defined. Notably, the nature of the antigen may be important in driving autoimmune pathology in lupus-prone mice. In burn wounds, clearing a plethora of self-antigens in necrotic tissue is an essential role of the macrophage and contributes to their hyperstimulated state.[26,52] Rapid clearance of apoptotic cells is essential to prevent intracellular leakage of toxic cell contents, additional inflammatory cascades and the shift from tolerance to immunity.[73–75] Macrophages from lupus-prone strains have been shown to have an apoptotic-dependent autoimmune phenotype that includes aberrant cytokine expression.[76] Importantly, non-autoimmune mice do not show this defect. Dysregulated functional activity (decreased phagocytosis, errors in self-Ag recognition and processing) and aberrant signaling events (cytokines, apoptotic ligands and receptors) involved with the clearance of apoptotic cells are thought to predispose an individual to autoimmune disease[14,76–81] Thus, effective clearance of apoptotic cells might be an active process of immune tolerance following a traumatic injury or 'danger signal' minimizing exposure to self antigens and the expansion of self-reactive effector T cells.

In summary, our research shows that traumatic injury can activate the SLE disease processes. The link between traumatic injury and the manifestation of SLE, along with the increasing numbers of female US military personnel deployed to military theatres, makes autoimmune diseases, like SLE, highly relevant to military populations. Improved understanding of the mechanisms triggering SLE and disease progression could lead to diagnostic and prevention strategies that would reduce the negative impact of SLE not only on individual warfighters but also on the hundreds of thousands of civilians stricken with this debilitating (and potentially life-threatening) disease. Finally, the wound healing injury model described here provides an excellent model for testing of novel therapeutic interventions.

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